CLINICAL AND VACCINE IMMUNOLOGY, Sept. 2009, p. 1378–1380 Vol. 16, No. 9
1556-6811/09/$08.00+0 doi:10.1128/CVI.00198-09

An Oral *Mycobacterium bovis* BCG Vaccine for Wildlife Produced in the Absence of Animal-Derived Reagents[∇]

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Received 17 May 2009/Returned for modification 17 June 2009/Accepted 24 June 2009

Cultures of *Mycobacterium bovis* BCG, comprising predominantly single-cell bacilli, were prepared in broth without animal-derived reagents. When formulated into a vegetable-derived lipid matrix, the vaccine was stable in vitro and was immunogenic in vivo upon feeding it to mice. This formulation could be useful for oral vaccination of wildlife against tuberculosis, where concern over transmissible prions may preclude the field use of vaccines containing animal products.

Many livestock diseases persist despite attempts at eradication. In the United Kingdom, Ireland, and New Zealand, bovine tuberculosis (TB) persists among farmed ruminants because the disease repeatedly spills over from adjacent wildlife species which harbor endemic TB, namely the European badger (*Meles meles*) in the former two cases and the Australian brushtail possum (*Trichosurus vulpecula*) in the latter case. Bovine TB is also a problem in North America, where wild cervidae are vectors of the disease to livestock (6). Hence, prophylactic vaccination of wildlife reservoir species is being pursued as a disease control strategy (6), which would be achievable by distributing an oral bait vaccine into the environment.

A prototype oral-delivery *Mycobacterium bovis* BCG-based vaccine has been reported to limit TB progression in trials on captive or free-ranging wildlife, as demonstrated upon *Mycobacterium bovis* challenge of vaccinated animals (1, 10, 11), and to prevent *M. bovis* infection following natural exposure to the pathogen (14). This vaccine comprises an edible lipid matrix into which live BCG has been formulated and evenly dispersed (1), which ensures that predominantly single-cell bacilli are released upon degradation of the matrix (e.g., by artificial detergents in vitro or by gastrointestinal tract processing in vivo). Upon in vivo oral delivery, released bacilli establish replicating populations in the lymph nodes of the alimentary tract, principally in the cervical lymph nodes (CLNs) draining the upper tract and the mesenteric lymph nodes (MLNs) draining the lower tract (3). Currently, the necessary high proportion of single-cell bacilli in the vaccine is achieved by formulating BCG that has been grown in liquid-phase culture using supplemented Middlebrook 7H9 broth (3). It is noteworthy that the broth supplements include bovine-derived products, namely serum albumin (as a carrier protein) and polysorbate (Tween 80; as a dispersant), which is a by-product of fractionated beef tallow.

In this regard, European and United Kingdom directives restrict the distribution of bovine-derived products into the environment (4, 5) due to concern over the release and propagation of prion diseases. We have conducted the present study specifically to address this concern with respect to the oral-delivery BCG vaccine. We herein report a source of BCG, grown in liquid phase as predominantly single-cell bacilli, using a modified 7H9 broth free of bovine-derived reagents. Furthermore, we describe the efficacy of this source of BCG when formulated into a vegetable-derived lipid matrix and fed as an experimental oral vaccine to mice, comparing these mice to mice which received a standard vaccine comprising BCG grown in conventional 7H9 broth and formulated into a lipid matrix of animal origin.

For this study, BCG strain Danish 1311 was utilized. As outlined in previous publications (1, 2), bacteria were cultured in Middlebrook 7H9 broth (Becton Dickinson, North Ryde, Australia) modified by the addition of alkalinized oleic acid and glucose. The standard version of this broth was additionally supplemented with 5 mg/ml bovine serum albumin (BSA) (Fraction V; Gibco Laboratories, Auckland, New Zealand) and 0.5 ml/liter standard tallow-derived Tween 80 (Sigma Products, Perth, Australia); the experimental broth preparation was instead supplemented with various concentrations of HyPep soy hydrolysate protein (commonly referred to as vegetable albumin; Gibco) and Tween 80 derived from the fractionation of vegetable fat (Sigma product number P6224-500ML). Bacterial growth was monitored via optical density measurements over 5 days until mid-log phase was achieved; bacilli were harvested from each preparation, and the number of CFU were enumerated by plating onto Middlebrook 7H11 agar. Dry smears of each BCG preparation were stained for acid-fast bacilli using Ziehl-Neelsen staining, and images were assessed microscopically via an Olympus DP70 camera and analyzed digitally using Olympus DP Controller version 2.2.1.227. About 100 acid-fast objects per smear were analyzed, and the average particle size and size distribution were calculated. Furthermore, samples of BCG grown in either the standard or the experimental broth were formulated into the vaccine delivery matrices Lipid-C (animal-derived fat) or Lipid-PK (vegetable-derived fat), respectively. Subsamples of the lipid-formulated vaccines were withdrawn at regular intervals to assess vaccine storage stability at ambient room temperature (by detergent extraction of live bacilli and plating onto 7H11 agar [3]). Finally, 1-ml standard or experimental vaccine samples were flavored with 10% coconut powder

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∇ Published ahead of print on 1 July 2009.

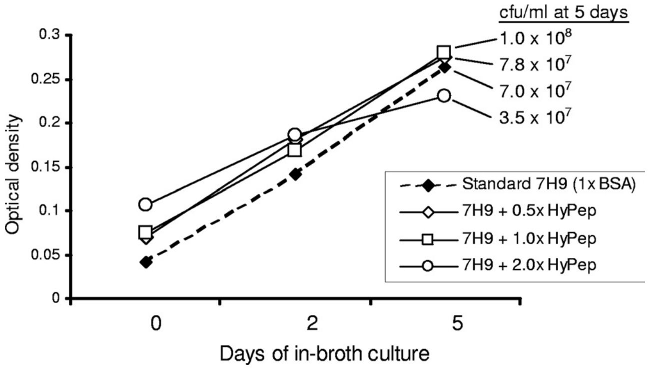

FIG. 1. In vitro growth characteristics of BCG grown under standard conditions (in 7H9 broth supplemented with 1× BSA, 5 mg/ml) compared to those of BCG grown under experimental conditions (in 7H9 broth supplemented with an equivalent 1× level of HyPep in place of BSA or with 0.5× or 2× HyPep).

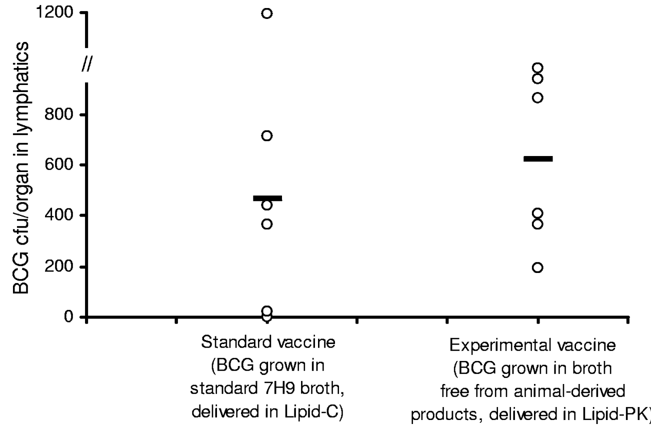

FIG. 3. BCG colonization of the alimentary tract lymph nodes (comprising MLNs and CLNs) of BALB/c mice for 8 weeks, following oral vaccination with either the standard vaccine (comprising BCG grown in BSA-supplemented 7H9 broth and formulated into Lipid-C) or the experimental vaccine (comprising BCG grown in broth free of animal-derived products and formulated into Lipid-PK). Bars represent group means from individual animals (open circles).

(Maggi Foods/Nestle Ltd., Rhodes, Australia) and offered on an individual basis to groups of 10-week-old female BALB/c mice as voluntary uptake oral vaccines (six animals/vaccine group); consumption of the entire vaccine dose by each mouse was confirmed by close examination of the individual cage for any sample wastage. Mice were subsequently euthanized 8 weeks postvaccination, and bacterial delivery and vaccine immunogenicity were determined as measures of vaccine success by assessing the BCG load in the alimentary tract lymphatics (combined MLN and CLN bacterial counts) and the splenic gamma interferon (IFN-γ) response to the *M. bovis* purified protein derivative (PPD-B; Prionics Inc., Switzerland) (8).

We found similar BCG growth rates when using HyPep vegetable protein in the experimental broth at an equivalent inclusion dose (1× HyPep = 5 mg/ml) or BSA in the standard broth (Fig. 1). Moreover, similar numbers of bacilli were recovered from the experimental and standard broths, with both preparations yielding $>10^7$ CFU per ml (Fig. 1). Both broth preparations contained a predominance of single-cell bacilli, as follows: in the standard BSA-supplemented BCG preparation, the mean Ziehl-Neelsen-stained/digitally imaged particle length obtained was 3.54 μm (range, 1.0 to 20 μm), with 71% of objects imaged as singlets; in the experimental HyPep-supplemented BCG preparation, the mean particle length obtained was 3.52 μm (range, 1.0 to 15 μm), with 67% of objects being singlets. Both preparations were thus considered appropriate for formulation into lipid matrices to produce vaccines. BCG that had been grown in broth free of animal-derived products, and formulated into Lipid-PK, showed in vitro storage stability characteristics similar to those of the standard vaccine (comprising BCG grown in standard 7H9 broth and formulated into Lipid-C), with both preparations maintaining potential delivery doses of $>5 \times 10^6$ BCG bacilli per vaccine dose after 6 weeks of storage at room temperature (Fig. 2). BCG colonization of the lymph nodes draining the alimentary tract was recorded in 5/6 mice which received the standard vaccine and 6/6 mice which received the experimental (animal product-free) vaccine (Fig. 3). Mice in both vaccine groups mounted significant IFN-γ responses following vaccination (Table 1). There were no significant differences in the magnitude of IFN-γ responses observed in mice receiving either the standard vaccine or the experimental vaccine (as determined using one-way analysis of variance of $\log_{10}$-transformed data).

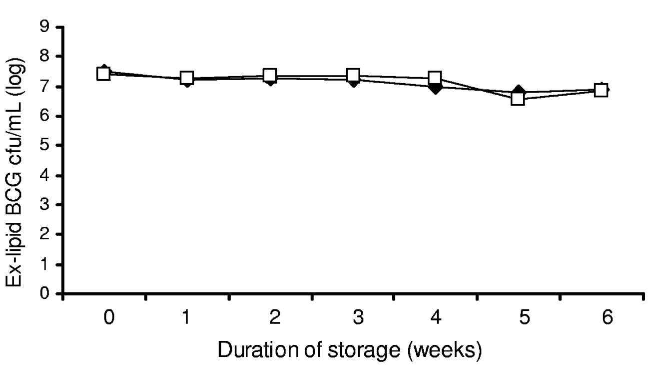

FIG. 2. In vitro 20°C storage characteristics of the standard vaccine (comprising BCG grown in BSA-supplemented 7H9 broth and formulated into Lipid-C) (□) compared to those of the experimental vaccine (comprising BCG grown in broth free of animal-derived products and formulated into Lipid-PK) (▲).

Animal-derived products that are in current or projected use for wildlife vaccines include meat- and fish-based baits for delivery of oral rabies vaccine (12) and an animal fat-derived lipid matrix for the delivery of oral TB vaccine (the latter containing BCG harvested from standard 7H9 culture broth supplemented with BSA and bovine tallow-derived Tween 80 [1, 2]). However, distribution of vaccine components derived from livestock animal sources into the environment raises concern, due to the theoretical transmission of transmissible spongiform encephalopathies (4, 5), among which the bovine form (bovine spongiform encephalopathy) is predominant. The current lipid matrix oral BCG vaccine circumvents this issue by utilizing BSA sourced from New Zealand that has been certified as bovine spongiform encephalopathy free (1–3). However, in this study, we investigated the novel approach of in-

TABLE 1. Vaccine immunogenicity in BALB/c mice tested 8 weeks following oral delivery of the standard or experimental vaccine

Type of vaccine[a]	Magnitude of IFN-γ response	
	No. of IFN-secreting cells (mean no. of cells/10^6 splenocytes ± SEM)	Amt of IFN secreted into culture supernatant (mean pg/ml ± SEM)
Standard vaccine (six mice/group)		
BCG in Lipid-C	201 ± 77	1,640 ± 1,281
Lipid-C only (control)	1.0 ± 0.7	20 ± 3
Experimental vaccine (six mice/group)		
BCG in Lipid-PK	213 ± 70	894 ± 353
Lipid-PK only (control)	1.7 ± 1.3	35 ± 7

[a] The standard vaccine comprises BCG grown in BSA-supplemented 7H9 broth and formulated into Lipid-C, while the experimental vaccine comprises BCG grown in broth that is free of animal-derived products and formulated into Lipid-PK.

stead eliminating all animal-derived products from the vaccine production process.

We were able to demonstrate similar growth characteristics for BCG when using 7H9 broth supplemented with HyPep and vegetable-derived Tween or with conventional BSA and tallow-derived Tween. Bacilli grown in either of these media for 5 days yielded $>10^7$ CFU per ml of culture broth and, upon harvest, appeared predominantly as single cells. This is an important consideration for an oral-delivery BCG vaccine, since while singlet bacilli readily gain access to the host's gut lymphatic system (3), mycobacteria are well known to form dense aggregates under unfavorable culture conditions, sometimes comprising thousands of cells and measuring over 100 μm (9), which may not be taken up so readily; experimental studies using graded-size polymer spheres have demonstrated that objects measuring >10 μm are effectively excluded from the host's gastrointestinal tract lymphatic system (13). As a practical wildlife vaccine, formulated BCG must also be amenable to near-deployment storage, and further studies herein identified similar in vitro storage characteristics for the experimental vaccine as well as for the standard vaccine when samples were stored under ambient (room temperature) conditions. One further component for a successful wildlife vaccine is that the delivery material either should be compatible with an existing oral-delivery bait or should form a bait in its own right; we have recently reported the latter case, since Lipid-PK is amenable to flavoring and invokes a high uptake rate in target wildlife species (possums) when appropriately flavored (7).

Importantly, both the standard vaccine and the experimental vaccine were shown to be capable of delivering live bacilli to the alimentary tract lymphatics of mice, following voluntary uptake of flavored vaccine samples. Both formulations were immunogenic, invoking significant systemic level IFN-γ responses. Interestingly, while the magnitude of the secretory IFN-γ response invoked using the standard vaccine was almost double of that of the experimental vaccine, responses were also more variable in this group; hence, overall there was no statistical difference in the responses invoked using either formulation. It is therefore likely that an oral-delivery BCG-based vaccine for wildlife can be produced in the absence of animal-derived products, if such is deemed necessary for regulatory purposes. Although the bait attractants that are employed in a working wildlife vaccine will vary depending on which wildlife species is being targeted (i.e., badgers or possums), it is a further consideration here that so long as lipid-compatible flavorings can be utilized for these species, the entire production and environmental distribution of the vaccine could feasibly be performed without the inclusion of animal-derived products.

We thank Yvonne Coughlan (Centre for Innovation) and Wenzel Czepluch (Department of Microbiology & Immunology), both at the University of Otago, New Zealand, for technical support.

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